

Bacterial Flora of the Hemolymph of the Blue Crab, *Callinectes sapidus*: Most Probable Numbers

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Received for publication 18 October 1974

The hemolymph of 290 freshly collected blue crabs from Chincoteague Bay, Va., was sampled over a 15-month period from August 1968 through November 1969 and most probable numbers of bacteria were determined by tube dilution. The hemolymph of 18% of all crabs sampled was found to be sterile, with 16% sterility in summer and 23% in winter samples. Despite individual variations, male crabs as a group had a higher bacterial hemolymph burden than females, and among both sexes summer counts were higher than winter. The hemolymph of crabs with missing appendages had significantly higher counts than uninjured crabs. The annual mean hemolymph most probable numbers per ml was 2,756 for males, 1,300 for females, and 1,876 for both sexes. The higher bacterial levels found in the hemolymph of male crabs may, in part, be explained by the fact that males, which predominated in the summer samples, had a higher incidence of injury and missing appendages than did females.

During field investigations of recurring summer epizootics among blue crabs, *Callinectes sapidus*, in Georgia and South Carolina, the hemolymph of moribund blue crabs was examined microscopically and by blood culture. The cause of the disease, characterized by a gray-colored abdomen and profuse bleeding, was then unknown. Although the etiology of "gray-crab" disease has now been ascribed to the ameboid protozoan, *Paramoeba perniciosa* (10), we discovered that the microbial complement of normal crab hemolymph was also unknown.

On the basis of their experience with vertebrates, most microbiologists would logically assume that the hemolymph of crabs is normally a sterile fluid. The circulating hemolymph is protected from the external environment by the epidermis (8) and an active clotting mechanism, which in health quickly seals bleeding from the loss of limbs and other wounds routinely experienced by these aggressive animals.

Bang and Krassner (Biol. Bull. Woods Hole, 115:343, 1958) and Krassner (Biol. Bull. Woods Hole, 125:382 to 383, 1963) found blood from the body cavity of the sipunculid worm, *Phascolosoma gouldii*, was sterile and had antibacterial activity, and Johnson and Chapman (4) reported comparable findings with the hemolymph of another sipunculid, *Dendrostomum zostericum*. In a study of the California sea

hare, *Aplysia californica*, a nudibranch mollusk, Pauley et al. (6) found the hemolymph to be normally sterile. Surveying Canadian Atlantic lobsters for the presence of the pathogen *Gaffkya homari* (*Aerococcus viridans* var. *homari*), Stewart et al. (1, 11) found *Gaffkya* in 5% and other bacteria in 19.5% of lobster hemolymphs. Since a phenyl ethyl alcohol medium selective for *G. homari* was employed for initial isolation, many hemolymph bacteria may have been inhibited by the selective agent.

Potential bacterial pathogens of public health significance have been documented in gills, viscera, and processed meat from healthy blue crabs and claws, and hemolymph of diseased crabs in commercial holding tanks (2, 5, 7, 12). This paper reports a seasonal survey of bacteria found in the hemolymph of freshly collected, commercially acceptable blue crabs from Chincoteague Bay, Va.

MATERIALS AND METHODS

Two hundred ninety blue crabs, 169 females and 121 males, were collected in crab pots or by dredge on 32 sampling dates between August 1968 and November 1969 from Chincoteague Bay, near Greenbackville, Va. Water temperatures ranged from 1 to 30 C and salinities from 28 to 35‰. The crabs were identified by sex and injuries and missing appendages were noted. (Crabs are traditionally gathered in bushel baskets and appendage losses are very common, either from fighting, autotomy or by forcible separa-

tion of individuals from clinging masses.) The crabs were bled aseptically by cardiac puncture through the intersegmental membrane between the posterior of the carapace and the abdomen, after the puncture site had been swabbed consecutively with tincture of iodine and 70% alcohol (Fig. 1a). A crab can be safely handled by firmly grasping the base of one of the 5th pleopods (swim fins) with the thumb braced against the carapace (Fig. 1b). The powerful claws will then be unable to reach the hands of the operator. Care was taken to avoid contamination by entry into the adjacent hepatopancreas and samples suspected of such contamination were discarded. A 2-ml sample of hemolymph was drawn from each crab and two 1-ml portions were added to screw-capped tubes containing 9 ml of M-plate count broth (Difco) (TGY) made up with filtered Chincoteague Bay seawater (28‰ salinity) as diluent, and 0.03% sodium polyanthenol sulfonate (Grobax, Roche Diagnostics, Nutley, N.J.). Three additional 10-fold serial dilutions were made from each portion, so that each sample consisted of a double array of four dilutions ranging from 10^{-1} to 10^{-4} . Inoculated tubes were observed for turbidity after 5 days of incubation at 22 C, and the most probable numbers (MPN) were determined from Hoskin's tables (3). For statistical purposes, hemolymphs scoring greater than MPN 6,600, beyond the range of the test, were arbitrarily considered to be 6,600. Probable significant differences among the MPN counts were determined using the Student's *t* test. Differences in group means were considered significant if $\alpha = 0.01$. The crabs were grouped by sex, condition, loss of appendages, season, and water temperature.

Plates were streaked from tubes of the highest dilutions showing growth; and from the plates, isolates representing the predominant organisms in the hemolymph were selected. Usually the highest dilutions proved to be pure cultures of single organisms. Isolates were collected for determinative studies (9).

RESULTS

On an annual basis the hemolymph of only 18% of all crabs sampled was found to be sterile. The annual mean hemolymph count (MPN) per ml was 2,756 for the males, 1,300 for the females, and 1,876 for both sexes (Table 1). Although large variations, from 0 to > 6,600 per ml of hemolymph, were found among individual crabs, significant differences between the means of groups were evident. The (121) male crabs demonstrated a mean MPN count of 2,756 per ml and higher average counts per milliliter of hemolymph than did the (169) females, which yielded a mean count of 1,300 per ml. The male-female ratio was not constant for all samples, since male crabs comprised the majority in summer samples, whereas females predominated in samples obtained in winter. Crabs with missing appendages (119 crabs with a mean count of 2,710 per ml) demonstrated a significantly higher count than did intact crabs (161 animals with a mean count of 1,673 per ml).

The seasonal variation in bacterial counts of

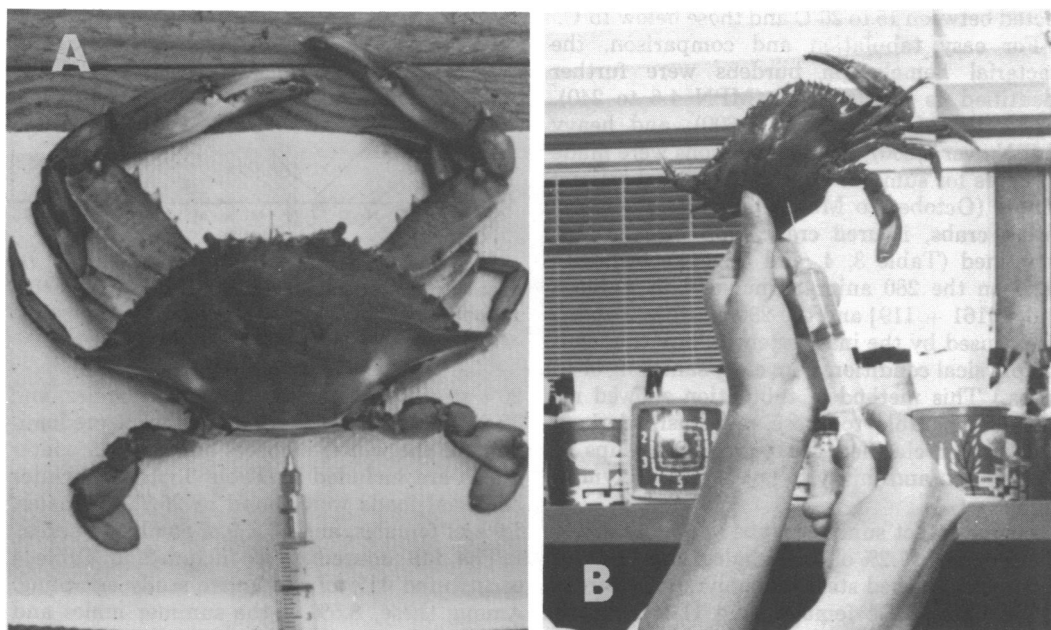


FIG. 1. (a) Needle is inserted into cardiac sinus through posterior intersegmental membrane. (b) Crab is held vertically by grasping swim-fin to facilitate bleeding and avoid injury to operator.

TABLE 1. Seasonal variations in bacterial counts (MPN) per milliliter of hemolymph

Determinants	Spring (March to May)	Summer (June to August)	Fall (September to November)	Winter (December to February)	Annual
Males					
No. of crabs	15	70	30	6	121
Arithmetic mean	3,042	2,424	3,400	3,344	2,756
Females					
No. of crabs	30	63	60	16	169
Arithmetic mean	2,018	1,870	1,405	14	1,300
Combined					
No. of crabs	45	133	90	22	290
Arithmetic mean	2,523	1,684	2,055	1,020	1,876

the crab hemolymph is summarized in Table 1. However, since seasonal variation implies variation in physical parameters, such as water temperature, the crabs were also compared by grouping the data for those animals collected when the water temperature was above 15, 20, or 25 C as opposed to those below 15, 20, or 25 C (Fig. 2). The bacterial count was significantly lower for samples collected at water temperatures below 15 to 20 C (Table 2). No significant difference in bacterial counts was noted for crabs collected at water temperatures >25 versus <25 C. It should be noted, however, that only two samples were collected when the water temperature was between 15 to 20 C. No significant difference was found among samples collected between 15 to 20 C and those below 15 C.

For easy tabulation and comparison, the bacterial hemolymph burdens were further identified as sterile, light (MPN 4.6 to 240), moderate (MPN 1,300 to 6,600), and heavy (MPN over 6,600), and comparisons were made by sexes for summer (April to September) and winter (October to March) samples including intact crabs, injured crabs, and both groups combined (Table 3, 4, and 5). (The disparity between the 280 animals included in Table 3 and 4 [161 + 119] and the 290 crabs in Table 5 was caused by the inadvertent failure to record the physical condition of an early sampling of 10 crabs.) This method of tabulation showed results comparable to those previously derived and further clarified the interrelationships of season, sex, and injury to levels of hemolymph flora.

Among intact summer crabs (Table 3) 22.7% of the males, 17.2% of the females, and 19.4% of both sexes showed sterile hemolymph, whereas 15.9% males, 7.8% females, and 11.1% of both sexes showed heavy bacterial burdens. Winter hemolymph levels for intact animals showed

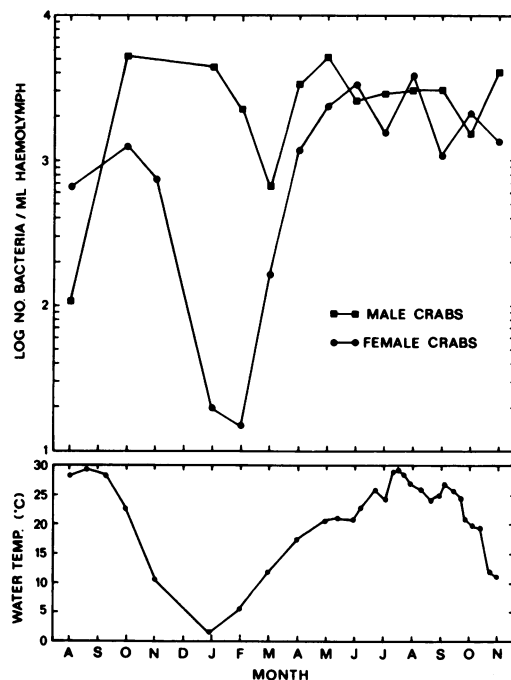


FIG. 2. Relationship of the bacterial count of the crab hemolymph with water temperature at the time of sampling. The data points represent an average of several sampling dates during the given month.

20% sterility for males, 34.2% for females, and 30.2% for both sexes. (Females were predominant in the winter samples and only 15 winter males are included in Table 3.) Heavy winter bacterial loads were found in 26.7% of males, 7.9% of females, and 13.2% of combined sexes.

The 119 injured crabs included in Table 4 constituted 41% of the entire study sampling. Among these, 8.5% of the summer males and 14.7% of the summer females, with a combined 11.1%, had sterile hemolymph, as compared to a

TABLE 2. *Relation of bacterial counts (MPN) per milliliter of hemolymph to water temperatures^a*

Determinants	15 C		20 C		25 C	
	Below	Above	Below	Above	Below	Above
No. of crabs	64	226	77	213	176	114
Arithmetic mean	1,272	2,136	1,382	2,184	1,960	1,911

^a The *t* values for 15, 20, and 25 C were 2.10, 2.04, and 0.13, respectively.

TABLE 3. *Bacterial hemolymph burden; intact crabs*

Determinant	Summer						Winter					
	Male		Female		Combined		Male		Female		Combined	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Sterile	10	22.7	11	17.2	21	19.4	3	20.0	13	34.2	16	30.2
Light	17	38.6	35	54.7	52	48.1	3	20.0	18	47.4	21	39.6
Moderate	10	22.7	13	20.3	23	21.3	5	33.3	4	10.5	9	17.0
Heavy	7	15.9	5	7.8	12	11.1	4	26.7	3	7.9	7	13.2

TABLE 4. *Bacterial hemolymph burden; injured crabs*

Determinants	Summer						Winter					
	Male		Female		Combined		Male		Female		Combined	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Sterile	4	8.5	5	14.7	9	11.1	3	20.0	4	17.4	7	18.4
Light	12	25.5	17	50.0	29	35.8	4	26.7	13	56.5	17	44.7
Moderate	11	23.4	4	11.8	15	18.5	2	13.3	2	13.3	4	10.5
Heavy	20	42.6	8	23.5	28	34.6	6	40.0	4	26.7	10	26.3

TABLE 5. *Bacterial hemolymph burden; all crabs*

Determinants	Summer						Winter						Annual rate	
	Male		Female		Combined		Male		Female		Combined		No.	%
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%		
Sterile	14	15.4	16	16.3	30	15.9	6	20.0	17	33.9	23	22.8	53	18.3
Light	29	31.9	52	53.1	81	42.9	7	23.3	41	57.7	48	47.5	129	44.4
Moderate	21	23.1	17	17.3	38	20.1	7	23.3	6	8.5	13	12.9	51	17.6
Heavy	27	29.7	13	13.3	40	21.2	10	33.3	7	9.9	17	16.8	57	19.7

combined 19.4% sterility for intact crabs during the same season (Table 3). Heavy bacterial burdens were found in 42.6% of the injured summer males and 23.5% of the females for a combined 34.6% for both sexes. A comparison with the combined value of only 11.1% for both sexes in intact animals emphasizes the conclusion that loss of appendages or other injuries enhances the probability of hemolymph bacteremia. Winter samplings showed similar but less marked results: 20% male sterility, 17.4% female sterility, 18.4% combined; and 40%

heavy infection for females, 26.7% for males and 26.3% for both sexes (compared to only 13.2% for intact crabs).

Table 5 combines the findings of both intact and injured crabs of both sexes for both seasons and summarizes these findings. In summary we found that on an annual basis the hemolymph of approximately 18.3% of commercially acceptable blue crabs from Chincoteague Bay was sterile whereas the hemolymph of about 19.7% of these crustaceans carried a relatively heavy bacterial flora.

The taxonomy of these bacteria is presented in the following paper (9).

DISCUSSION

It is generally assumed that the circulatory system of healthy animals is sterile, whereas the presence of bacteria is usually considered to be a sign of disease. However, only 18% of the 290 crab hemolymph samples examined in this study were found to be sterile. In fact, 31% of the hemolymph samples revealed MPNs of 6,600 per ml or greater. By comparison, in an examination of the hemolymph of 2,035 lobsters, Stewart et al. (1, 11) found the hemolymph sterile in about 75%; but as the authors stated, the bacteria isolated "do not necessarily reflect the actual predominance of types in the hemolymph, since the medium was selective and the test qualitative."

The effects, if any, of naturally occurring bacteremia of the blue crab are unknown. Differences in bacterial counts between male and female crabs were observed. However, careful scrutiny of the sample sets indicates that differences in the counts observed between sexes may not be directly sex linked. Male crabs comprised more than 50% of summer samples. Since the bacterial counts were generally higher for both sexes during the warmer months and more male than female crabs were collected during this period, a rise in the mean population of bacteria associated with male crabs is not unexpected. Higher bacterial counts were also found among crabs missing one or more appendages, suggesting that bacteria may gain entry to the hemolymph through wounds left by the loss of a limb. This entrance portal is available only for a short period, since in healthy crabs clots form quickly with little loss of blood (8). Male crabs were more likely to be missing appendages (51%) than were females (33%). Male crabs might, therefore, be expected to carry higher bacterial populations in their hemolymph. Indeed, a comparison of the bacterial counts of the hemolymph of the intact female crabs

compared with the intact males revealed a slight, but not statistically significant, difference.

A bacterial population exists in the hemolymph of a majority of market blue crabs. Generally, hemolymph bacterial counts were found to be higher in male crabs, crabs with missing appendages, and crabs collected during the summer. On an annual basis about 18% of the hemolymphs were sterile.

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